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A novel method for hydrophobin extraction using CO_2 foam fractionation system

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ABSTRACT

Due to the exceptional properties and many potential applications of hydrophobins, special fungal proteins, it becomes necessary to develop a real scale procedure for their production and purification. In our previous study (Deckers et al., 2010) [CO_2–hydrophobin structures acting as nanobombs in beer, Brew. Sci. 63:54–61], the strong interaction of CO_2–hydrophobin was demonstrated. For the first time, in an approach to isolate hydrophobin HFBII from the growth media of *Trichoderma reesei*, a foam fractionation system using CO_2 as the sparging gas was investigated in this study. Using CO_2 foam fractionation, the concentration of HFBII was increased from 0.10 ± 0.02 mg/mL up to 0.57 ± 0.04 mg/mL. This was shown after a purification step by conventional liquid chromatography and identification of the goal protein using MALDI-TOF. The obtained molecular weight of the protein was 7.042 kDa which corresponds to the complete molecule of HFBII, minus the last aminoacid. Micro-spectrophotometry was used for quantification of purified HFBII. Moreover, different parameters of the foam fractionation system were optimized. The concentration of the protein after treatment by CO_2 followed by liquid chromatography was increased from 0.32 ± 0.02 to 0.44 ± 0.06 mg/mL when the flow rate of gas injection was changed in the range of 1–3 L/min. The highest amount of HFBII equal to 0.57 ± 0.04 mg/mL was obtained by the highest ratio of liquid height over the column height. Using the larger pore size frits causes increased protein absorption as well. The gushing potential of samples revealed that in contrast to the samples before CO_2 treatment, interestingly, no gushing was observed for the samples after treatment. The possibility that stable aggregates of HFBII molecules are formed as a consequence of their high concentration is discussed in this paper. By using DLS analysis of the overfoam, 100 nm particle size of CO_2 nanobubbles coated by HFBII was obtained. The final concentration of the protein was carried out using Amicon® ultracentrifuge device with the average recovery of $63.8 \pm 8.2\%$.

1. Introduction

Hydrophobins are a large family of cysteine rich proteins synthesized by fungi with a molecular mass of around 10 kDa (Cooper and Kennedy, 2010). They are secreted into liquid media by fungi or present on the surface of aerial mycelia (Stubner et al., 2010). They can assemble at a hydrophilic–hydrophobic interface forming an amphipathic film (Linder, 2009). A broad spectrum of functions in fungal growth and development is related to these proteins. Based on the self-assembly, hydrophobins are divided into two classes: I and II (Wessels, 1994). Molecular aggregates formed in these two classes are distinguished on the basis of solubility and morphology.

Thanks to their extraordinary properties, hydrophobins were suggested for a number of applications (Khalesi et al., 2012). Many of them involve the adsorption of hydrophobins to modify surfaces (Janssen et al., 2002). The self-assembly of hydrophobins (Wosten and Wessels, 1997) makes them interesting for using as stabilizers of emulsions (Wosten et al., 1994), foaming agents (Hektor and Scholtmeijer, 2005) and targets for the immobilization of other components (Linder, 2009). The application of hydrophobins in biosensor developments (Bilewicz et al., 2001), and in tissue engineering (Janssen et al., 2002) were studied as well. Therefore, developing methods to detect, isolate and purify these valuable proteins in real scale is interesting for industries, especially those which are looking for products in the area of nano-biotechnology.

One of the class II hydrophobins with exceptional characteristics, is HFBII. The structure of HFBII shows a rigid and amphiphilic molecule (Linder et al., 2005). This explains its surface activity and the formation of supra-molecular assemblies. It was demonstrated that HFBII could reduce the air/water surface tension down to 30 m Nm^{-1} (Cox et al., 2007). However, more knowledge is required to fundamentally understand the surface behavior of HFBII.

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Therefore, it becomes important to study the required parameters for production, isolation and purification stages at larger scale.

Foam fractionation of substances is an adsorptive bubble separation method for enriching diluted surface-active substances dissolved in water (Maruyama et al., 2006). This accessible technique has been studied in many fields, such as chemical/biochemical engineering, analytical chemistry, and wastewater treatment (Maruyama et al., 2007). In principle, the strong amphipathic nature of proteins and enzymes, with polar and non-polar groups, causes them to be preferentially adsorbed at the gas–liquid interface and foam fractionation can be used to separate and to concentrate such proteins (Aksay and Mazza, 2007). The adsorption at the gas–liquid interface in this system lowers the surface tension and enhances bubble formation. Moreover, the molecules form an elastic film around the bubbles increasing foam stability (Linke et al., 2005). The rich foamate is then collected. The enhanced protein concentration in the foam is due to the combined effect of interfacial adsorption and foam drainage, and it is proposed that adsorption occurs only while the bubbles rise in the liquid pool (Bhattacharjee et al., 1997). This technique is simple and has significant potential for lowering the high costs of protein recovery (Tseng et al., 2006).

The type and the flow rate of gas injection, the ratio of the column height to initial liquid height, the pore size of the sintered glass disk, the initial liquid volume as well as the initial surfactant concentration are essential parameters which may cause different influences on performance of foam fractionation. Sarachat et al. (2010) demonstrated that by increasing the air flow rate, biosurfactant recovery was increased significantly, but the enrichment ratio was reduced. Similarly, Chana et al. (2007) reported that both the foam volume and the recovery percentage of the proteins in wastewater samples increased exponentially when increasing the air flow rate, while the enrichment ratio was decreased. Likewise, high feed flow rate results in low enrichment factors as the higher mass of surface-active molecules stabilizes the foam, thus increasing the volume of collected foamate (Merz et al., 2011). Consequently, the stability of the foam decreases leading to liquid holdup reduction. Another important factor which can affect the separation process is pH. Obviously, the isoelectric point is the optimum condition to enhance the separation (Linke et al., 2007). Despite the recent advances in foam fractionation, delivering both high biosurfactant recovery and high enrichment ratio at the same air flow rate is still impossible. Therefore, depending on the objective, the parameters have to be chosen.

During this study, we developed a simple, inexpensive method to produce, concentrate and purify hydrophobin HFBII using a foam separation technique with CO_2 as sparging gas. This is based on the knowledge of the strong interaction of CO_2 with hydrophobins, which by the way causes gushing problem in beer (Deckers et al., 2010). Gaining a better understanding of the HFBII–CO_2 interaction in such a system is another objective.

2. Experimental

2.1. HFBII production

Hydrophobin HFBII was produced using *Trichoderma reesei* MUCL 44908 (purchased from BCCM-BCCM-/MUCL (Agro)Industrial Fungi and Yeast Collection company) in a medium with lactose as carbon source described by Bailey et al. (2002) (Table 1).

The pH of the medium was adjusted to 4.5–5 by using HCl and heat sterilized. For inoculation, mycelium was collected from surface cultures of *T. reesei* on petri dishes with the medium of Malt Extract Agar (MEA) and subjected into the test tubes containing fresh medium culture. These were used as the overnight culture. After 12 h, the overnight cultures were added into the fresh medium with the ratio of 1:3 for further growth. Fungal growth was initiated at 25 °C, in a 2-L working volume fermenter (KGW-type 7174) with stirring and temperature control. After 7 days the medium was centrifuged (Beckman CouLter™, 8000 *g*) at 6 °C for 25 min and the supernatant was directed to the foam fractionator.

Table 1
The amount of different components in 1-L medium culture.

Component	Amount (per L)
Lactose monohydrate	41.33 g
Peptone	4.00 g
Yeast extract	1.00 g
KH_2PO_4	4.00 g
$(NH_4)_2SO_4$	2.80 g
$MgSO_4{\cdot}7H_2O$	0.60 g
$CaCl_2$	0.60 g
$CoCl_2{\cdot}6H_2O$	4.00 mg
$MnSO_4{\cdot}H_2O$	3.2 mg
$ZnSO_4{\cdot}7H_2O$	6.9 mg
$FeSO_4{\cdot}7H_2O$	10 mg

2.2. Design of the foam fractionation device and optimization procedure

The foam fractionator consisted of a glass column (ID = 52 mm) as shown in Fig. 1. For foaming, the CO_2 passed through a sintered glass disk. The collapsed foam was collected and stored for further analysis.

Gas flow rate, ratio of the liquid height over the column height as well as frits' pore size were optimized for the improvement of the CO_2 foam fractionation system. For gas flow rate, three different amounts of CO_2 flow were tested; 1, 2 and 3 L/min. In all cases, G_3 frit was used. The ratio of the liquid height over the column height was adjusted to 0.23. Three different ratios of the liquid height over the column height (0.13, 0.23, 0.33) and 3 different frit sizes (G_3,

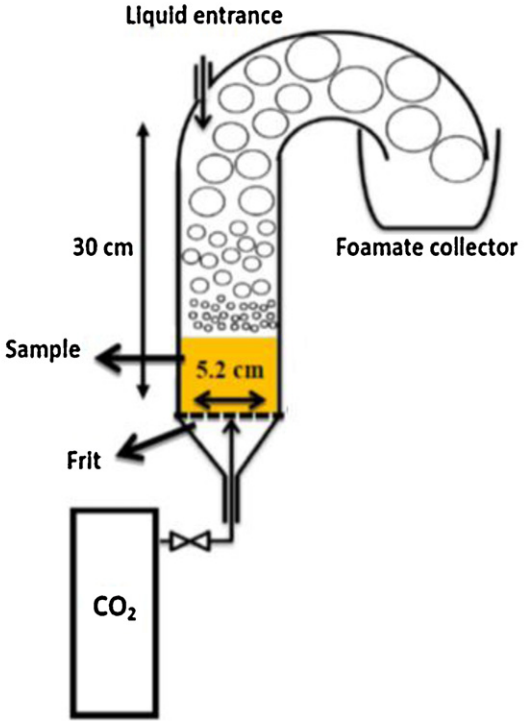

Fig. 1. Schematic diagram of CO_2 foam fractionation system.